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(54) METHOD FOR PRODUCING A PROTEOLYTIC ENZYME PREPARATION

(57) The invention relates to a method of preparing a proteolytic enzyme preparation which can potentially be used for the chemotherapy of diseases of humans, for the degradation of protein-containing compositions and for the digestion of complex substrates in the microbiological industry. In particular, the invention relates to a method for producing a novel proteolytic enzyme preparation using a microorganism which has so far not been used for the production of enzymes of the proteinase type. The microorganism strain ZIMET 43 647 is denominated as Nocardiopsis dassonvillei due to its taxonomic characteristics. The object of the invention is the isolation of a proteolytic enzyme preparation with fibrinolytic, biological slurry-clarifying properties as well as the ability to digest polypeptide-containing substrates of the fermentation industry to expand the range of such biocatalysts. The problem is solved by building the enzyme using aerobic submerse fermentation of a microorganism in media with appropriate C N sources and mineral salts, by concentrating using methods of ultrafiltration techniques in the culture filtrate, and optionally by lyophilizing in the conventional manner.

Title of the invention

Method for producing a proteolytic enzyme preparation

Technical field of the invention

The invention relates to a method for producing a proteolytic enzyme preparation which, due to its fibrinolytic characteristics, is of use for human medicine, due to its proteolytic action, is of use for the clarification of biological slurry and for the digestion of complex substrates in sewage water as well as the microbiological industry. In particular, the invention relates to the isolation of a proteinase using a microorganism which has so far not been known as proteinase builder.

Characteristics of the known technical solutions

Numerous representatives of the genera Bacillus, Aspergillus and Streptomyces are able to synthesize proteinases under suitable fermentation conditions, the proteinases differing with respect to their proteolytic characteristics on serine (E. C. 3. 4. 21), cysteine (E. C. 3. 4. 22), aspartic (E. C. 3. 4. 23) and metallo proteinases (E. C. 3. 4. 24). The known proteinase builders frequently produce mixtures of proteinases, e.g. in the present case alkaline, and neutral proteinases are produced simultaneously. On the other hand, the type of optimum temperature and optimum pH of known proteolytic preparations limit their applications.

For particular application purposes it is desirable that the proteolytic activity of the enzyme preparation under 30°C is very low whereas at temperatures of from 35 to 70°C it develops completely. The use of mesophilic representatives of the species Nocardiopsis as proteinase builder has not been known up to now. It is also not known that proteolytic enzyme preparations from Nocardiopsis having such a high optimum temperature, which is otherwise known only of thermophilic microorganisms such as thermoactinomyces, were obtained. Fermentations with the latter are characterized by a disadvantageous ratio of energy input to energy output.

Object of the invention

The invention serves to produce a proteolytic enzyme preparation with a high optimum temperature with the aid of a microorganism which, due to its mesophilic properties, is to be preferred over thermophilic microorganisms from an energetic viewpoint. Thus, the

listed disadvantages of the processes known so far for producing proteolytic enzyme preparations should be avoided and the range of such preparations should be broadened by a representative with a broad field of application.

Disclosure of the character of the invention

The object underlying the invention is to provide a technologically simple method allowing to produce a proteolytic enzyme preparation with a high optimum temperature from easily accessible and economical ingredients with high yield and without using thermophilic microorganisms.

According to the invention, this object is solved by culturing the microorganism described below or variants thereof under aerobic and sterile culture conditions in a liquid culture medium with carbon and nitrogen sources as well as with mineral salts.

The microorganism applied in this process was deposited with the depository institution for microorganisms, ZIMET, Zentralinstitut für Mikrobiologie und experimentelle Therapie der Akademie der Wissenschaften der DDR, 69 Jena, Beutenbergstraße 11, under the registration number ZIMET 43 647. The strain ZIMET 43 647 is assigned to the species Nocardiopsis dassonvillei (Brocq-Rousseu) Meyer (Int. J. Syst. Bacteriol. 26 (4): 487 to 493 (1976)). It was isolated from molding straw. The strain is aerobic, gram positive and katalase-positive. It has the cell wall chemotype III (meso-diamino pimelic acid. Arabinose and galactose are lacking). Mycolic acids and mandurose are lacking. The substrate mycelium develops well at 28°C on the organic medium 79 (Prauser and Falta. 1968. Z. Allg. Mikrobiol. 8 (1): 39 to 46) and on the ISP media 2, 3, 4 and 5 (Gottlieb und Shirling. 1966. Int. J. Syst. Bacteriol. 16 (3): 313 to 340). It is from almost colorless to slight yellowish-brownish (medium 79). Substrate mycelium hyphae being on the surface in the majority of cases grow outwards rectangularly to the border of the colony. More or less rectangular branchings start therefrom. Soluble pigments are missing in the agar. Air mycelium is best built on medium 79. It is mealy, white with a slight yellowish-greyish tinge. On other media the air mycelium is only developed at the borders of the colonies, or air mycelium hyphae can only be recognized using a microscope. The air mycelium hyphae fragment into prolately elliptic spores with even surface. Strain ZIMET 43 647 does not form melanoid pigments. It reduces nitrate, peptonizes milk, fluidizes gelatine, hydrolyzes starch and degrades xanthine and aesculin. The strain grows on D-glucose, D-mannose, maltose, D-mannit, D-fructose, sucrose and glycerine. It does not grow on L-arabinose, D-xylose, L-rhamnose, lactose, raffinose, adonite, dulcite and meso-inositol. The growth on arabinose, xylose, lactose and rhamnose, which is missing contrary to the

description of Meyer (loc.cit.), does not give reason at the present state of the taxonomic examination of the genus Nocardiopsis to describe another species within that genus.

Cultivation of strain ZIMET 43 647 as well as mutants or variants thereof proceeds under aerobic conditions. Mycelium fragments lyophilized in glucose-gelatine are inoculated in suitable solid agar media and subsequently in liquid, presterilized culture media, and the resulting mycelium is cultured in a manner known per se at a temperature of from 25 to 37°C (preferably 28°C) for a time of from 2 to 10 days (preferably 6 days) at acidity which is between pH 6.5 and pH 7.2 at the beginning of the fermentation process. The culture medium consists of carbon and nitrogen sources as well as inorganic salts. Starch, glucose, glycerol, mannit, dextrine, sucrose, soy oil and soy flour may be used as carbon sources. Apart from the nitrogen-containing substrates mentioned above, dry yeast, meat peptone and casein can be considered as nitrogen sources.

Good results can be obtained when adding mineral salts. The latter favor the progression of the fermentation depending on the culture medium used. In complex media containing different flours the addition of calcium carbonate, sodium nitrate and/or potassium phosphate is advantageous. The fermentation of the builder may be carried out in flasks with steep breast ("Steilbrustflaschen") and round-bottom flasks of different contents, in a glass fermenter as well as in V2A tanks.

The determination of the proteolytic activity of the crude culture broth of the strain ZIMET 43 647 of the species Nocardiopsis dassonvilei and of the enzyme preparation E-ZIMET isolated thereof was carried out according to the UV spectroscopic method described by M. Kunitz 1946 (J. Gen. Physiol., 29, 149). The isolation of the proteolytic enzyme preparation is carried out in that the culture solution is separated into mycelium and culture filtrate in the conventional manner, the culture filtrate is concentrated by means of ultrafiltration techniques and subsequently lyophilized.

The lyophilized novel enzyme preparation is a brownish, amorphous substance which is well dissolvable in water. The proteolytic enzyme preparation was examined for its protein degrading effectiveness on different substrates. The preparation can be characterized by the following substrate profile:

Substrates	Proteolytic action of E-ZIMET 43 647
Gelatine	+
Casein	+
Bovine serum albumin	+
Gamma globulin	-
Collagen	+
Fibrin	+

The optimum temperature of the proteolytic enzyme preparation E-ZIMET 43 647 ranges between 35 and 70°C (preferably at 60°C) if casein and/or diazo-casein are used as substrates at pH 7.6(Fig. 1). The optimum pH ranges between pH 6.0 and pH 10.0 (preferably pH 9.0) if a temperature of 60°C was chosen (Fig. 2).

The thermostability of the proteolyitc enzyme preparation E-ZIMET 43 647 was examined at 35 and 60°C and pH 9.0, for a period of 6 hours. It was shown that a 2-hour preincubation of the preparation at 35°C did not cause any loss in activity whereas a 6-hour preincubation resulted in a loss of the proteolytic activity to 90%.

In contrast thereto, after a 2-hour preincubation of the preparation at 60°C and after a 6-hour preincubation decreases in proteolytic activity to 35% and to about 8% were observed, respectively (Fig. 3).

Fresh fermentation solution of the builder ZIMET 43 647 as well as the enzyme preparation E-ZIMET 43 647 isolated thereof have specific properties to solubilize and/or partially hydrolyze proteins from animals and plants as well as from humans. Different application fields in agriculture, industry and human medicine result therefrom.

The proteolytic action of the preparation E-ZIMET 43 647 on gelatine was determined using the method described by Täufel et al. (J. Chromat., 93, 487 to 490, 1974). The action of the enzyme preparation on casein was determined using the method of Kunitz (J. Gen. Physiol., 29, 149, 1946). Furthermore, the proteolytic activity of the enzyme preparation was also examined on diazo casein according to a common procedure (J. Biol. Chem. 171, 501, 1947). The proteolytic action of E-ZIMET 43 647, present or lacking, was also measured with the substrates bovine serum albumin and gamma globulin using tyrosine release determined UV-spectroscopically according to the method of Kunitz. The proteolytic action of the enzyme preparation on collagen was proven using the hide powder azur technique. At the hide powder azur each free hydroxyl group of collagen is linked to a pigment residue so that a huge number of pigment molecules (Remazol Brilliant Blue) dissolves if a single molecule is cleaved off enzymatically. Thus, hide powder azur acts as "amplifier substrate" (Rinderknecht et al., Clin. Chim. Acta, 21, 197 to 203, 1968).

The fibrinolytic activity was determined using the fibrin agar plate technique according to Astrup and Müllertz (In: Gerinnungslaboratorium in Klinik und Praxis (Edit. E. Perlick and A. Bergmann, Verlag Georg Thieme-Leipzig, 1971, p. 374)).

Examples

1. As inoculation material for the inoculation of pre-culture mycelium fragments of Nocardiopsis dassonvillei, strain ZIMET 43 647, lypholized in glucose gelatine are used. A liquid culture medium of the following composition is suited for pre-culture medium:

Glucose	1.5 %
Soy meal	1.5 %
CaCO3	0.1 %
NaCl	0.5 %

Tap water. Sterilization: 35 min. at 115°C. Acidity between pH 6.5 and pH 7.0

The amount for inoculation of 80 ml pre-culture medium in a 500 ml flask with steep breast consists of 1 ml resuspended mycelium fragment lyophilizates. After pre-culture at 28°C on a shaker table with a vibration frequency of 180/min for 2 days, 80 ml of production culture are inoculated in 500 ml flasks with steep breast with 8 ml pre-culture.

A liquid culture medium with the following composition is suited for production medium:

Sucrose	0.25 %
$NaNO_3$	0.2 %
K_2HPO_4	0.1 %
$MgSO_4 * 7H_2O$	0.05 %
KCl	0.05 %
$FeSO_4$	0.001%

Aqua dest. Sterilization: 35 min. at 115°C acidity between pH 7.2 and pH 7.8

The duration of fermentation is between 6 and 10 days at 28°C on a shaker table with a vibration frequency of 180/min, before the maximum proteolytic activity is reached.

2. Proceed as in Example 1 with the exception that the production medium has the following composition:

Soy meal	2.0 %
Glucose	2.0 %
CaCO3	0.3 %
NaCl	0.5 %

In tap water. Sterilization: 35 min at 115°C. Acidity at pH 6.5.

3. The procedure differs from the one of Example 1 in that the inoculation material used for the inoculation of the pre-culture is grown on a solid agar medium having the following composition for a period of 10 days:

Glucose	1.0 %
Peptone	1.0 %
Yeast extract	0.2 %
Casamino acids	0.1 %
NaCl	0.6 %
Agar	1.5 %

Tap water. Sterilization: 35 min at 115°C. Acidity at pH 7.0.

4. The procedure differs from the one of Example 1 in that the 1st pre-culture is designed as described in Example 1 but subsequently the 2nd pre-culture is grown by inoculating 400 ml of the pre-culture medium in 2 l glass flasks with 10 ml suspension of the 1st pre-culture for a period of 24 hours.

400 ml of the 2nd pre-culture thus obtained serve for the inoculation of 20 l of the production medium described in Example 1, which is contained in a 32 l glass fermenter. During fermentation, which is carried out at 28°C at a stirring rate of 400 r/min and an air supply of 15 l/min, the foam formation can be controlled by adding small amounts of sunflower oil or polysiloxan-containing foam protection agents. The activity of the proteolytic enzyme obtained after a fermentation period of approximately 120 hours amounts to 0.9 to 1.4 µmol released tyrosin/ml in the supernatant of the culture solution of the wildtype strain.

5. After separating the mycelium from the fermentation solution obtained according to Example 4 by means of a separator, 20 l culture filtrate are concentrated to 3.5 l using ultrafiltration. For ultrafiltration a self-made apparatus is used which is equipped with cellulose acetate membranes type UF 1. The concentration factor amounts to 5.7. The proteolytic activity produced per 1 ml native culture solution effects the release of 0.9 µmol tyrosin in a casein solution at +60°C after 20 minutes. In contrast thereto, the proteolytic activity per ml concentrate amounts to 4.0 µmol released tyrosin under the same conditions. The concentration factor with respect to the proteinase activity amounts to 4.4. The enzyme yield after concentration amounts to 77.7% of the starting

solution. The filtrate current density reached a value of 16 l per hour and m^2 and the selectivity

$$\varphi = (1 - \frac{\text{C filtrate}}{\text{C starting solution}}) \times 100\%$$ amounts to 67%.

After ultrafiltration the proteolytic enzyme preparation is dried by lyophilization and stored at -20°C.

Invention claim

Process for producing a proteolytic enzyme preparation microbiologically characterized in that the microorganism Nocardiopsis dassonvillei, strain ZIMET 43 647, is cultured under aerobic conditions in liquid culture media containing carbon and nitrogen sources as well as mineral salts at a temperature of from 25 to 37°C during a time period of from 5 to 10 days, and the proteolytic enzyme E-ZIMET 647 thus built in the culture filtrate is concentrated using ultrafiltration techniques and, optionally, subsequently lyophilized.

To this 3 pages of drawings

Influence of the temperature on the proteolytic activity of E-ZIMET 43647

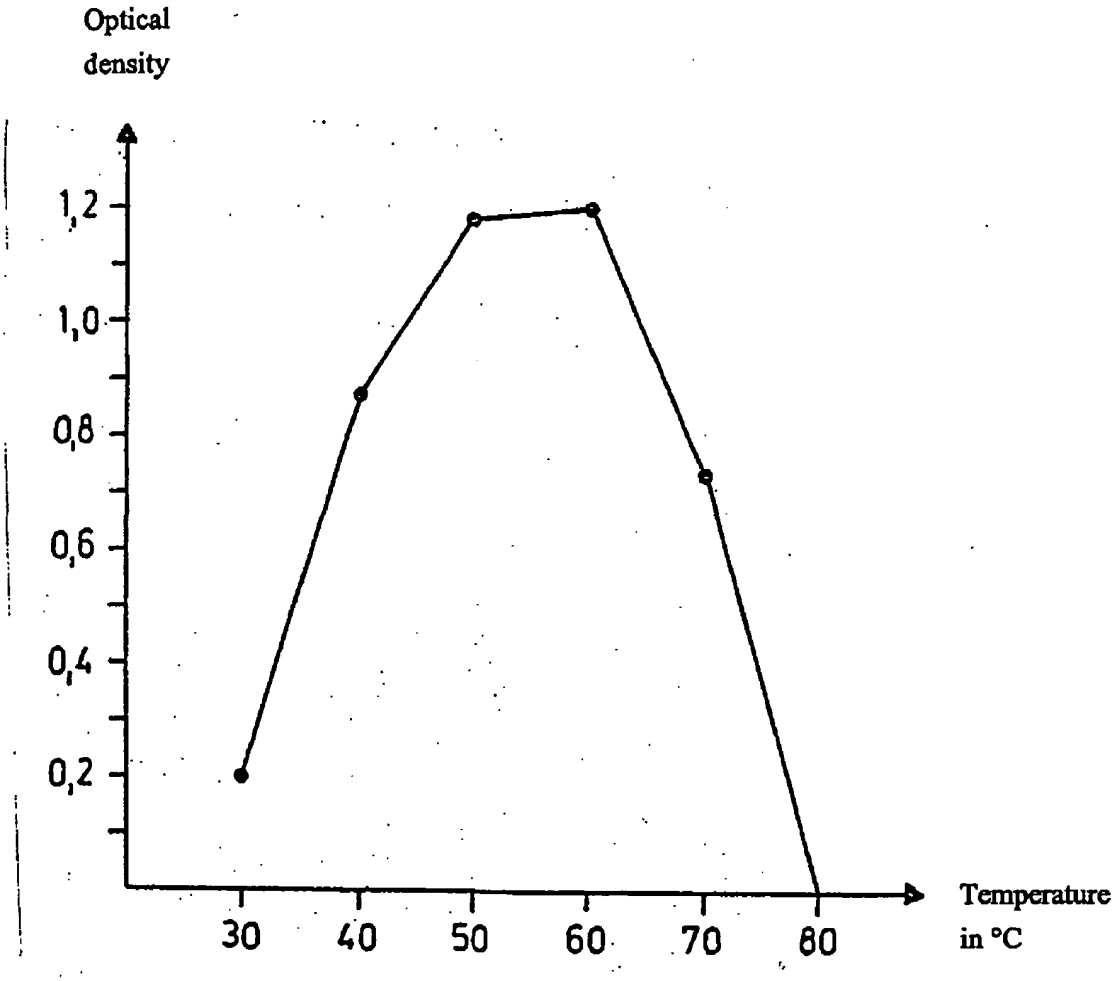

Fig. 1 Temperature action optimum of E-ZIMET 43637

Influence of the pH value on the proteolytic activity of E-ZIMET 43647

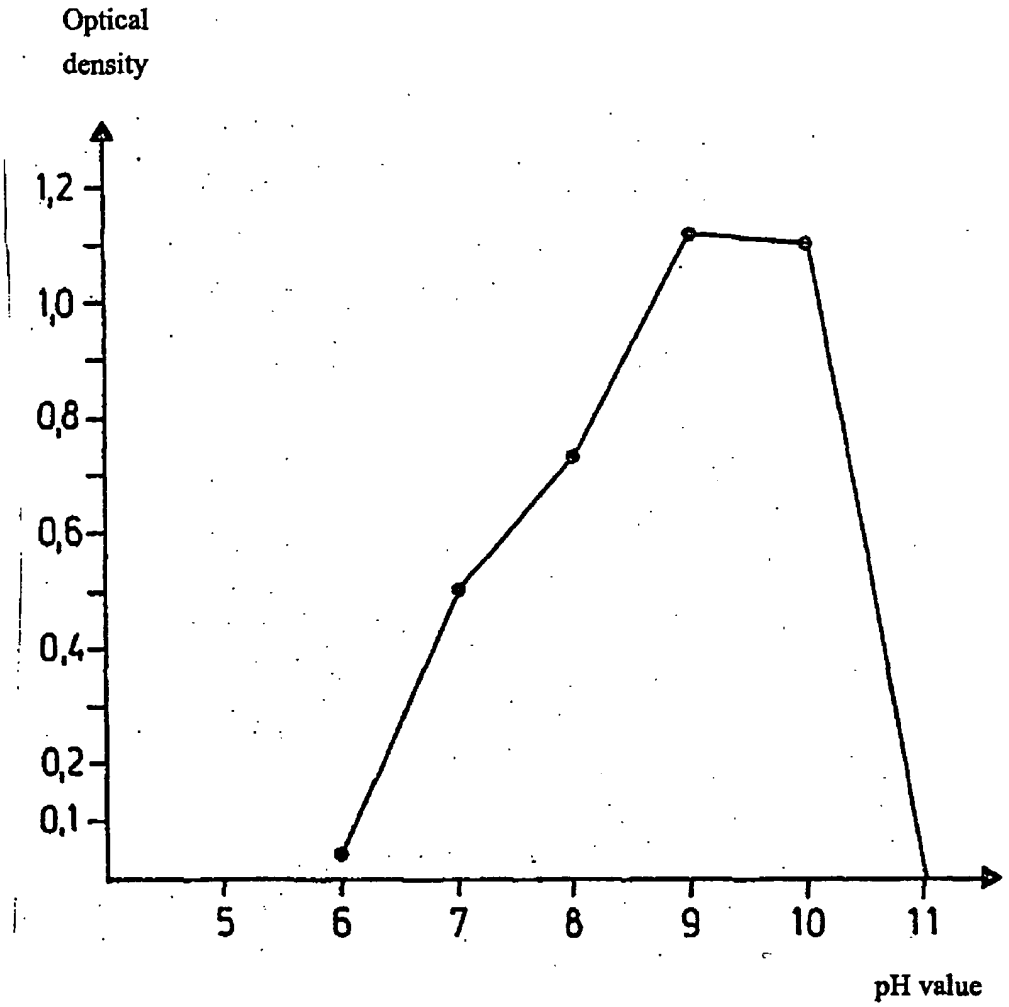

Fig. 2 pH action optimum of E-ZIMET 43637

Thermostability of E-ZIMET 43647
At 60°C —— and 35°C -------

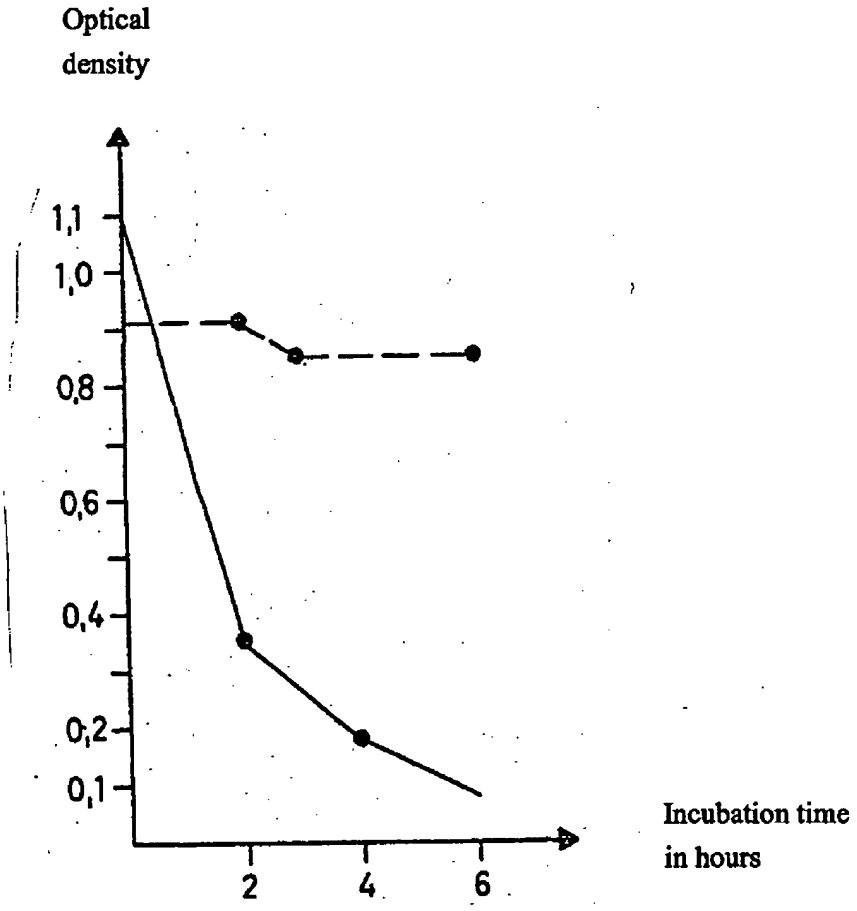

Fig. 3 Influence of the temperature on the activity of E-ZIMET 43637